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Regulatory Relationship among *piwi*, *pumilio*, and *bag-of-marbles* in *Drosophila* Germline Stem Cell Self-Renewal and Differentiation

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Summary

The transition from a *Drosophila* ovarian germline stem cell (GSC) to its differentiated daughter cell, the cystoblast, is controlled by both niche signals and intrinsic factors. *piwi* and *pumilio* (*pum*) are essential for GSC self-renewal, whereas *bag-of-marbles* (*bam*) is required for cystoblast differentiation [1–8]. We demonstrate that Piwi and Bam proteins are expressed independently of each other in reciprocal patterns in GSCs and cystoblasts. However, overexpression of either one antagonizes the other in these cells. Furthermore, *piwi;bam* double mutants phenocopy the *bam* mutant. This epistasis reflects the niche signaling function of *piwi* because depleting *piwi* from niche cells in *bam* mutant ovaries also phenocopies *bam* mutants. Thus, *bam* is epistatic to niche Piwi, but not germline Piwi function. Despite this, *bam*$^-$ ovaries lacking germline Piwi contain approximately 4-fold fewer germ cells than *bam*$^-$ ovaries, consistent with the role of germline Piwi in promoting GSC mitosis by 4-fold [3]. Finally, *pum* is epistatic to *bam*, indicating that niche Piwi does not regulate Bam-C through Pum. We propose that niche Piwi maintains GSCs by repressing *bam* expression in GSCs, which consequently prevents Bam from downregulating Pum/Nos function in repressing the translation of differentiation genes and germline Piwi function in promoting germ cell division.

Results and Discussion

Here, we investigate the regulatory relationships between Piwi, Bam, and Pum, three key regulators of GSC versus cystoblast fates. Among them, Pum and Bam are intrinsic factors [1, 4–8], whereas Piwi is expressed both in niche cells as an essential component of niche signaling and in GSCs to promote its division [2, 3]. Pum was originally identified as a maternal effect protein that heterodimerizes with NANOS (Nos) to bind and suppress the translation of its target *hunchback* mRNA in the posterior of the *Drosophila* embryo (reviewed in [9]). In addition, Pum and Nos have important germline development zygotic roles, including their cell-autonomous function for GSC maintenance [4, 5, 10]. In contrast to this function of Pum and Nos, Bam is necessary and sufficient in promoting GSC differentiation, even though its molecular activity is not known [6–8]. *bam* encodes two protein isoforms: the cytoplasmic (Bam-C) and the fusomal (Bam-F) forms, with Bam-C specifically present in cystoblasts and differentiating cysts but absent in GSCs [6, 7]. Finally, Piwi is the founding member of the evolutionarily conserved Piwi protein family (a.k.a. ARGONAUTE family) involved in stem cell division, RNA interference, transcriptional gene silencing, and other developmental processes [1, 2, 11–15]. In the *Drosophila* ovarian germline, Piwi is a nuclear protein that is preferentially expressed in GSCs but is only weakly expressed in cystoblasts and mitotic cysts, consistent with its germline function [3].

Piwi and Bam Are Expressed Independently of Each Other in Reciprocal Patterns in GSCs and Cystoblasts

To investigate the regulatory relationship between *piwi* and *bam*, we first confirmed the reciprocal expression pattern by double immunofluorescence microscopy of wild-type germaria for Piwi and Bam-C. As previously reported [3], a fully functional myc-tagged Piwi is expressed at high levels in GSCs and is downregulated in cystoblasts and early mitotic cysts (Figure 1A). In contrast, Bam-C is absent from GSCs but accumulates in most cystoblasts and mitotic cystocytes in germarial region 1 (Figure 1B). The downregulation of Piwi coincides with the zone of Bam-C expression (Figure 1C). In a few cases, we observed germ cells expressing both Piwi and Bam-C in cystoblast positions. These cells might represent the transitional stage from GSCs to cystoblasts. At a very low frequency, cystoblast-like cells express low levels of Piwi, but no detectable Bam-C. On the basis of *piwi;bam* double mutant analysis (see below), these cystoblast-like cells are likely to be undifferentiated or potentially apoptotic. Overall, the reciprocal expression pattern of Piwi and Bam-C proteins supports the opposing functions of *piwi* and *bam* genes.

To determine whether this reciprocal expression pattern is a result of mutually negative regulation toward each other's expression, we analyzed Bam expression in *piwi* mutants and vice versa. Because *piwi* mutant ovarioles typically contain germaria that are depleted of germline cells [1, 2], it is difficult to assay Bam-C expression in them. For this reason, we generated *piwi*^{1} GSC clones with the FLP-DFS (flipase-mediated dominant female sterile) technique ([16]; see Experimental Procedures). Bam-C is expressed normally in cystoblasts and early mitotic cysts in germaria that contain only *piwi*^{1} germline cells (Figure 1D). Moreover, no ectopic Bam expression was detected in GSCs. Therefore, proper Bam-C expression in the adult germline during oogenesis does not require *piwi*$^+$ function in the germline. To address whether *piwi* expression in apical somatic cells affects *bam* expression in GSCs, we eliminated Piwi in somatic niche cells. This was achieved by using *Yb* mutations that eliminate Piwi expression

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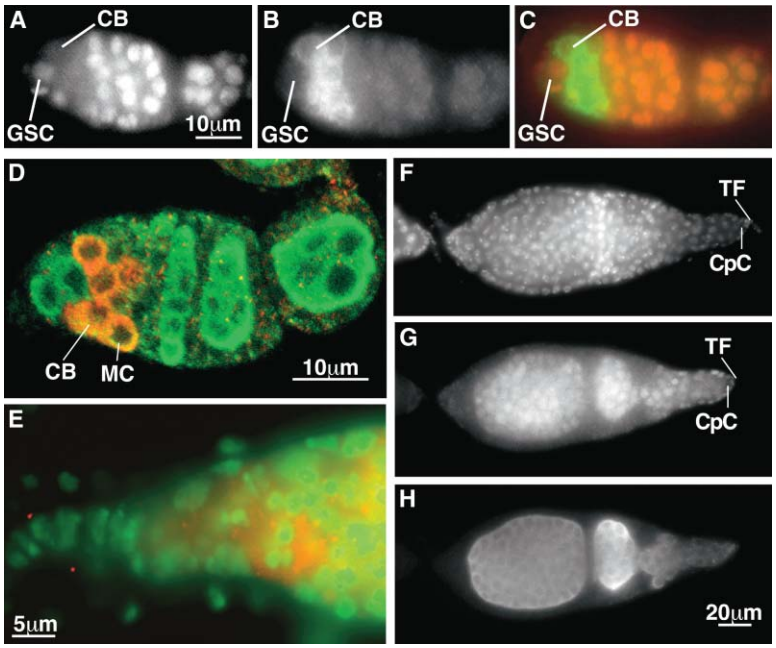

Figure 1. Piwi and Bam-C Are Expressed Independently of Each Other in Reciprocal Patterns in GSCs and Cystoblasts

(A–C) Reciprocal expression patterns of Piwi and Bam. Wild-type germarium stained for myc-Piwi (A) and Bam-C (B) is shown. Piwi is expressed in GSCs and late mitotic cysts but is downregulated in cystoblasts (CB) and early mitotic cysts. Bam-C is absent in GSCs but is abundantly present in Cb and early mitotic cysts. (C) is the merged image of (A) and (B). The bar in (A) indicates magnification for (A–C).

(D–H) Piwi and Bam expression are independent of each other. (D) A germarium containing *piwi*^{1} germline clones is stained for the germ cell marker VASA (green) and for Bam-C (red). Bam-C is expressed in *piwi*^{1} cystoblasts (CB) and early mitotic cysts (MC). (E) The anterior part of a *Yb* mutant germarium double labeled for Bam-C (red) and DAPI (green). Bam-C expression is unaffected in *Yb* mutant ovarioles. (F–H) *piwi* is expressed in *bam* mutants. A *P[myc-piwi];bam*$^{\Delta 86}$/*bam*$^{\Delta 86}$ ovariole stained with DAPI (F), anti-Myc antibody (G), and anti-VASA antibody (H) is shown. Note that *myc-piwi* is expressed in all germ cells and apical somatic cells. The bar in (H) indicates magnification for (F–H).

specifically in niche cells [17, 18]. *Yb* mutants are phenotypically very similar to *piwi* mutants. However, if examined within the first day of eclosion, *Yb* mutant germaria still contain germ cells. As shown in Figure 1E, Bam-C expression is unaffected in adult *Yb* mutant ovaries, suggesting that there is no specific requirement for YB or Piwi in niche cells for proper Bam-C expression or localization in the germline. Taken together, the above analyses indicate that neither niche nor germline *piwi* is required for Bam-C expression.

We next investigated whether the absence of Bam affects Piwi expression. A *P[myc-piwi]* transgene was introduced into a *bam* null mutant background to monitor Piwi expression. Ovaries were dissected from these females and stained with Myc antibody to monitor the Piwi expression and with VASA antibodies to label germ cells. Piwi is present in all *bam* null germline cells (Figures 1F–1H). In addition, Piwi is also strongly expressed in apical somatic cells that correspond to terminal filament, cap cells, and inner sheath cells in the wild-type germarium (Figures 1F–1H). Therefore, *bam*$^{+}$ function is dispensable for Piwi expression in the germline and the apical somatic cells.

Ectopic Bam Expression Downregulates Piwi Protein in GSCs

We then tested whether *piwi* or *bam* negatively regulates the expression of the other. Previously, we reported that overexpression of Piwi in apical somatic cells increases the number of GSC-like cells. These ectopic stem cell-like cells fill regions 1 and 2a of the germarium and, thus, displace Bam-C-expressing cells to region 2b [3]. If Piwi and Bam expression are mutually antagonistic, the prediction would be that expanding Bam expression to GSCs would downregulate Piwi expression there during oogenesis. To express Bam-C protein ectopically in GSCs, we used a heat shock-inducible *bam* transgene that places the *bam* cDNA under the control of the *hsp70* promoter [7]. Flies carrying a *P[myc-piwi]* and a *hs-bam* transgene were subjected to heat shock twice daily for 3 days after eclosion (see Experimental Procedures). Ovaries were subsequently dissected and stained for Bam-C and Myc to monitor ectopic Bam-C expression and its effects on Piwi expression. As predicted, ectopic expression of Bam in GSCs diminished Piwi expression specifically in these cells (Figures 2A–2C). Interestingly, ectopic Bam expression in both somatic cells and other germline cells within and beyond the germarium had no effect on Piwi expression in these cells (Figures 2A–2C; data not shown). Particularly, Piwi expression in apical somatic cells (i.e., cap cells and inner sheath cells) of the germarium was unaffected by ectopic Bam expression. In control flies lacking the *hs-bam* transgene, we observed no defects in Piwi expression after heat shock treatment (data not shown). This indicates that ectopic Bam expression may specifically downregulate the germline Piwi expression.

To determine whether downregulation of germline Piwi after ectopic *bam* expression is an indirect consequence of *bam* converting GSCs to cystoblasts or, alternatively, reflects a more direct interaction between the two genes, we subjected the *P[myc-piwi];hs-bam* flies to only a single 2 hr heat shock, followed by a 6 hr recovery period before ovaries were dissected. We estimated that this period should be sufficient for Bam protein to be expressed and to antagonize Piwi without allowing GSCs sufficient time to completely switch their fate. The dissected ovaries were stained for α-spectrin and Myc to monitor the effects of ectopic Bam-C expression on Piwi expression (Figures 2D–2F). Ectopically expressed Bam-C elicits a rapid response in the downregulation of *myc-piwi*. Expectedly, not all germline cells

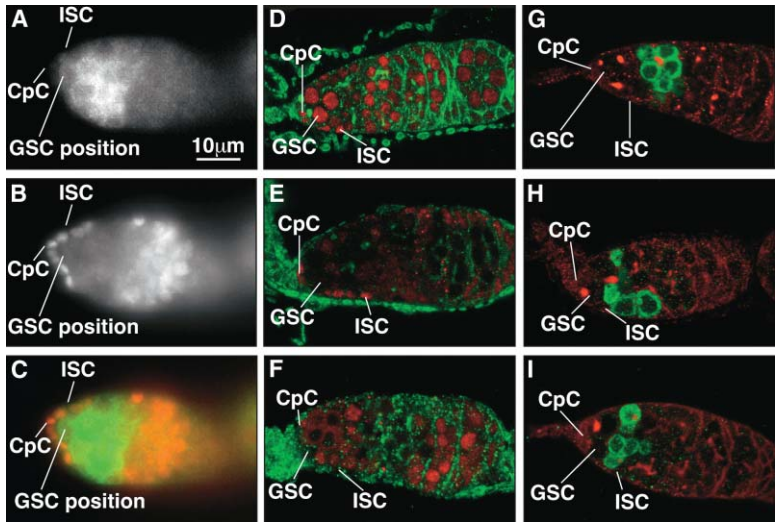

Figure 2. Ectopic Bam Expression Downregulates Piwi Level in GSCs

(A–C) The effects of long-term ectopic Bam overexpression. (A) Anti-Bam-C staining of the germarium reveals ectopic Bam expression in the GSC position. (B) Anti-myc-Piwi staining reveals that ectopic Bam expression sharply downregulates myc-Piwi level in germ cells in the GSC positions. However, ectopic Bam expression has no effect on Piwi level in somatic cap cells (CpC) and inner sheath cells (ISC). (C) is the merged image of (A) and (B).

(D–F) The short-term effects of a single pulse of ectopic Bam overexpression. *hs-bam;myc-piwi* flies were heat shocked at 37°C for 2 hr, dissected 6 hr later, and stained with Myc antibodies (red) and spectrin antibodies (green). (D) A non-heat shocked control showing the normal nuclear expression of myc-Piwi is shown. (E) and (F) show rapid downregulation of myc-Piwi expression in early germ cells (E) and, less frequently, myc-Piwi relocation to the cytoplasm (F), after ectopic Bam expression.

(G–I) A single pulse of ectopic Piwi overexpression shows no short-term effects on Bam-C expression. Piwi was overexpressed either in both soma and germline by a *hsp70-myc-piwi* transgene (G and H) or only in the soma by *hsp70-gal4/piwi*^{EP} transgenes (I). Bam-C expression was monitored with a Bam-C::GFP protein fusion transgene [27]. Flies were heat shocked as in panels (D)–(F) and stained with anti-1B1 (red) and anti-GFP (green) antibodies. (G) A non-heat shocked control showing the normal Bam-C:GFP transgene expression is shown. A single pulse of somatic (I) and germline (H) overexpression of Piwi shows no effect on Bam-C:GFP transgene expression within 6 hr. The bar in (A) denotes the image scale for all panels.

responded equally strongly or rapidly. Nevertheless, Piwi in GSCs was almost always downregulated. In contrast, Piwi expression in niche cells was almost not affected, whereas Piwi in other early germ cells displayed mixed responses. Interestingly, some cystoblasts showed a cytoplasmic localization of myc-Piwi (Figure 2F) rather than its normal nucleoplasmic localization. This change in myc-Piwi localization might reflect an intermediate state of Piwi between its nuclear localization and its degradation in the cytoplasm.

Flies carrying a *bam:GFP* transgene together with a *hs-myc-piwi* or *piwi*^{EP}*;hs-gal4* transgene were heat shocked and allowed to recover as described above and then stained with anti-1B1 and anti-GFP antibodies (see Experimental Procedures) to test whether overexpression of Piwi affects Bam expression. Expression of *bam:GFP* was unaffected when Piwi was overexpressed either in niche cells (Figure 2I; *piwi*^{EP}*;hsgal4*) or in all cells (Figure 2H; *hs-myc-piwi*). These results, combined with previous findings that Piwi overexpression displaces Bam expression posteriorly within the germarium [3], suggest that either downregulation of Bam-C by Piwi requires more time or that it is an indirect consequence of GSC expansion upon Piwi overexpression.

bam Is Epistatic to *piwi*

The mutually independent expression of Piwi and Bam does not, however, rule out their regulatory relationship in GSC cell fate, whereas the suppression of *piwi* in GSCs by ectopic *bam* expression suggests that these two genes interact antagonistically. To further define the interaction between *piwi* and *bam*, we constructed females lacking both *piwi* and *bam* function and analyzed the double mutants' ovaries. In contrast to the *piwi* mutant phenotype, in which ovarioles typically contain a germlineless germarium and 2–3 egg chambers (Figure 3A), the double mutant ovaries are characterized by "tumorous" germaria filled with hundreds of undifferentiated germ cells (Figure 3C). Moreover, there is no apparent egg chamber development in the double mutant ovary (Figure 3C). This phenotype is qualitatively similar to the tumorous phenotype observed in *bam* mutant ovaries, which can contain up to thousands of undifferentiated germ cells (Figure 3B). The *piwi;bam* double mutant phenotype therefore indicates that *bam* is epistatic to *piwi*. Given the opposing functions of *piwi* and *bam*, these results suggest that *piwi* acts upstream of *bam* to repress its function in promoting GSC differentiation.

Although the *piwi;bam* double mutant shows a *bam*-like phenotype, there is a difference between the defect of the double mutant and that of *bam* alone. The *bam* mutant typically contains 300–1000 undifferentiated germ cells, whereas the *piwi;bam* double mutants contain only 50–300 germ cells. One possible explanation for this difference is the absence of the mitosis-promoting, germline cell autonomous *piwi* function in the double mutant. The cell autonomous function of *piwi* in GSCs is to promote mitosis, resulting in a 4-fold increase of mitotic rates [3]. In *bam* mutants, "tumorous" germ cells are more mitotic because of the presence of *piwi*$^{+}$ function, whereas in *piwi;bam* double mutants, "tumorous" germ cells are less mitotic because of the absence of *piwi*$^{+}$ function. Therefore, these analyses suggest that, whereas *bam* is epistatic to the niche function of *piwi*, the cell autonomous function of *piwi* is epistatic to *bam*.

The Epistasis of *bam* over *piwi* Reflects Piwi Signaling Function from Niche Cells

To verify the complex epistasis between *bam* and distinct somatic versus germline functions of Piwi, we in-

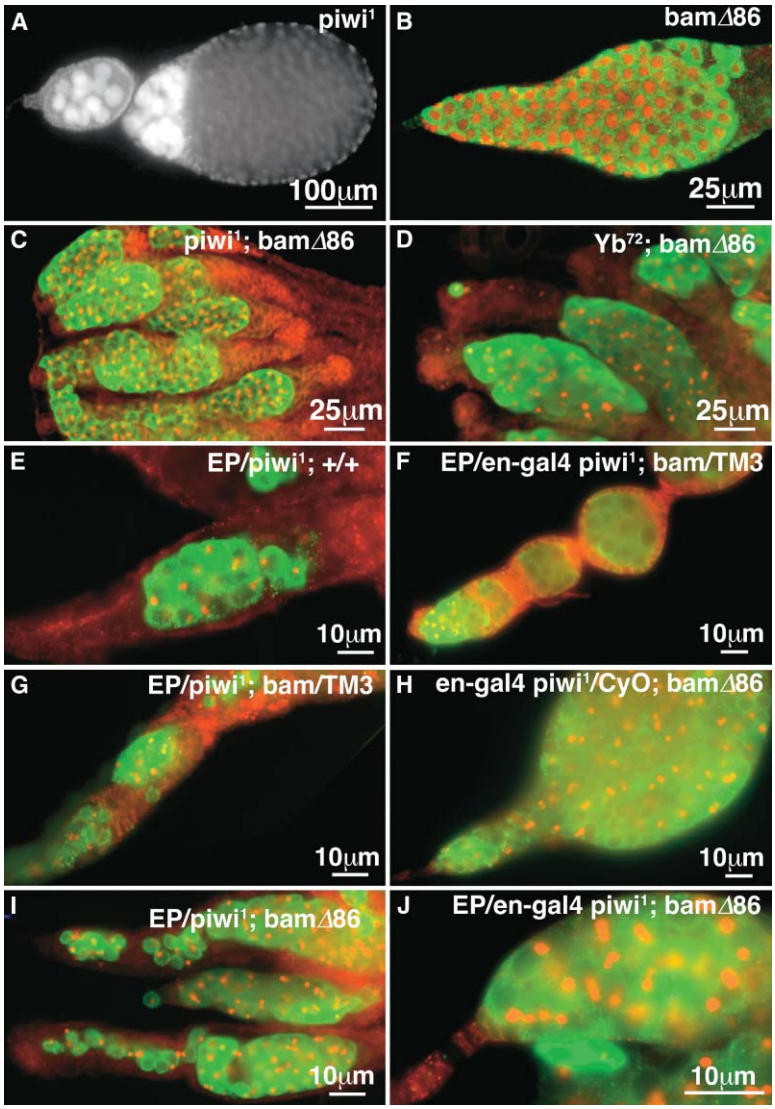

Figure 3. Epistasis Analysis between *piwi* and *bam* Mutants

(A–C) *piwi;bam* double mutants display a *bam*-like phenotype. (A) A $piwi^{1}$ mutant ovariole containing a germline-depleted germarium (Ge) connected to two egg chambers representing the products of the two differentiated GSCs as revealed by DAPI nuclear staining is shown. (B) A tumorous *bam* mutant ovariole containing 300–1000 undifferentiated germ cells as revealed by staining for VASA (green) and myc-Piwi (red). Note that myc-Piwi is present in the nucleus of all undifferentiated *bam* mutant germ cells, suggesting that these germ cells are GSC-like (also see Figure 5). (C) *piwi;bam* double mutant ovariole characterized by mildly tumorous germaria filled with 50–300 undifferentiated germ cells with no apparent egg chamber development, as revealed by double labeling for VASA (green) and 1B1 (red).

(D–J) *bam* is epistatic to *piwi* function in niche cells but not in germ cells. All panels show 1B1 (red) and VASA (green) staining. (D) High magnification images of a *Yb;bam* double mutant ovariole are shown. (E and G) $piwi^{EP}/piwi^{1}$ heteroallelic ovaries display a *piwi* mutant phenotype. (I) $piwi^{EP}/piwi^{1};bam^{\Delta 86}/bam^{\Delta 86}$ displays *bam* phenotype. (F) *en-gal4* restores PIWI expression in anterior somatic cells by activating $piwi^{EP}$ in those cells. (H) *en-gal4* alone does not alter *bam* phenotype. (J) *en-gal4*,$piwi^{1}/piwi^{EP};bam^{\Delta 86}/bam^{\Delta 86}$ displays a mild *bam* phenotype.

vestigated the effect of specifically removing Piwi protein from either the germline or the somatic niche cells of *bam* mutants. The *piwi* (somatic)*;bam* double mutant was achieved by generating *Yb;bam* double mutants because *Yb* specifically eliminates *piwi* expression in niche cells [18]. The *piwi* (germline)*;bam* double mutant was achieved by driving transgenic *piwi* expression specifically in the niche cells of a *piwi;bam* double mutant background.

Yb;bam double mutant ovaries display a clear *bam* phenotype (Figure 3D). This phenotype, however, is not as attenuated as in *piwi;bam* double mutants, but rather appears to be as pronounced as in *bam* single mutants. This result supports the assumption that the epistasis of *bam* over *piwi* reflects the somatic *piwi* function, and the attenuated *bam* phenotype of the double mutant reflects the germline cell autonomous *piwi* function.

To further verify this hypothesis, we analyzed the phenotype of *piwi* (germline)*;bam* double mutants. The *piwi* (germline) mutant was generated with an *en-gal4* transgene to drive the expression of $piwi^{EP}$ to produce specific expression of fully functional Piwi in niche cells. Because $piwi^{EP}$ is inserted into the *piwi* locus, it is therefore a *piwi* mutant allele in the absence of *gal4* expression (Figures 3E and 3G). We generated the *en-gal4* $piwi^{1}/piwi^{EP}$ transheterozygotes in *bam* mutant and wild-type backgrounds. The *piwi/* $piwi^{EP};bam^{+}$ ovaries display the expected *piwi* mutant phenotype (Figures 3E and 3G). The *en-gal4* $piwi^{1}/piwi^{EP};bam^{\Delta 86^{-}}/TM3$ *Sb* ovaries appear wild-type, aside from a mild reduction in size, and give rise to females capable of laying eggs (Figure 3F). This finding directly confirms that Piwi expression in niche cells is sufficient for GSC maintenance, whereas the observed reduction in ovary size may reflect the absence of germline *piwi* function in promoting GSC mitoses. As expected, the *en-gal4* $piwi^{1}/CyO;bam^{\Delta 86}/bam^{\Delta 86}$ flies display typical *bam* mutant ovarioles (Figure 3H). Also as expected, $piwi^{1}/piwi^{EP};bam^{\Delta 86}/bam^{\Delta 86}$ and *en-gal4* $piwi^{1}/piwi^{EP};bam^{\Delta 86}/bam^{\Delta 86}$ ovaries display the phenotypes of *piwi* (somatic)*;bam* double mutants and the *piwi* (germline)*;bam* double mutants, respectively (Figures 3I and 3J). These analyses further verified that *bam*

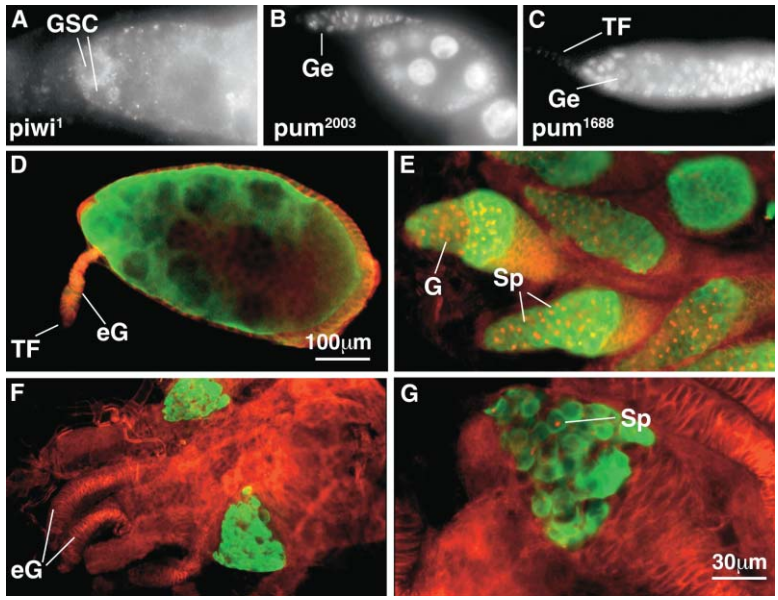

Figure 4. Regulatory Relationship between *piwi* and *pum*

(A–C) Piwi and Pum expressions are independent of each other. (A) A $piwi^{1}$ mutant germarium shows normal Pum expression GSCs. (B and C) pum^{2003} and pum^{1688} mutant ovarioles show normal myc-Piwi expression in the germarium (Ge) and in nurse cells of mutant egg chambers, as well as in terminal filament (TF) and other somatic cells of the germarium. (D–G) Germ cell proliferation in *bam* mutants requires Pum. (D) pum^{ET1}/pum^{ET9} mutant ovarioles characterized by germline-empty germaria (eG) and an egg chamber. (E–G) Ovaries are double stained with anti-VASA (green) to label germ cells and anti-1B1 (red) antibodies to label spectrosomes and outline somatic cells. (E) $bam^{\Delta 86}$ mutant germaria (G) are filled with proliferating GSC-like germ cells containing spectrosomes (Sp). (F) Low magnification image of *pum,bam* double mutants in which the majority of the germaria are devoid of germline, except that a few contain a small number of germ cells. (G) High magnification image of a *pum,bam* double mutant germaria containing a few proliferating germline cells. Images in (D)–(F) are at the same magnification.

is epistatic to somatic niche *piwi* function, yet germline *piwi* is epistatic to *bam* function.

The Expressions of Piwi and Pum Are Independent of Each Other

The fact that Piwi expression in somatic cells has a downregulating effect on Bam-C expression in GSCs [3] raises the question of how this signal may be relayed. The reciprocal expression patterns of Piwi and Bam-C in the germline closely resemble those of Pum and Bam-C. Pum maintains GSC self-renewal during oogenesis [1, 4], whereas Bam promotes GSC differentiation [6]. GSCs are depleted in *pum* mutants but overproliferate in *bam* mutants. This raised the possibility that Piwi may exert its functions by acting on Pum. We therefore explored Pum expression in $piwi^{1}$ mutants and Piwi expression in pum^{1688} and pum^{2003} mutants. The expression of one gene was not detectably altered in the mutant background of the other (Figures 4A–4C), suggesting that neither gene regulates the other's expression. However, *pum* encodes two distinct protein isoforms (156 kDa and 130 kDa). Either isoform is sufficient for maternal function, but both are required for zygotic function, including GSC maintenance [4]. Because pum^{1688} and pum^{2003} eliminate the expression of the 156 kDa and 130 kDa Pum isoforms, respectively, these results could suggest that either the 156 kDa or the 130 kDa isoform of Pum alone is sufficient for proper germline Piwi expression. Even if this is the case, the niche expression of *piwi* is independent of *pum* because *pum* is not required somatically to maintain GSCs [4].

The Proliferation of Germ Cells in *bam* Mutants Requires Pum Function

To more definitively determine the regulatory relationship between Pum and the Piwi-Bam-C pathway, we constructed and analyzed *pum,bam* double mutants (see Experimental Procedures). If somatic Piwi acts through Pum to regulate Bam-C, then *pum,bam* double mutants should resemble *piwi;bam* double mutants. This, however, was not the case. In $pum^{ET1},bam^{\Delta 86}/pum^{ET9},bam^{\Delta 86}$ double mutant flies, in which both pum^{ET1} and pum^{ET9} are null alleles, the majority of germaria were devoid of germ cells (>90%; Figure 4F). Only a minority of germaria (<10%) contained a number of undifferentiated germ cells with restricted proliferation (Figure 4G). This range of defects is indistinguishable from that of the phenotype of typical *pum* mutant ovaries [1, 4] (Figures 4B–4D). These results suggest that *pum* is epistatic to *bam* and that the proliferation of germ cells in *bam* mutants requires Pum function.

Conclusions

In summary, our results show that somatic niche Piwi function antagonizes Bam-C, which in turn antagonizes Pum and germline Piwi. The niche function of PIWI in downregulating BAM function appears to converge with the Dpp signaling pathway that is also required for GSC maintenance [19, 20] (Figure 5). This is based on the following observations: First, the expression of *dpp* does not require *piwi* (A.S. and H.L., unpublished data). Therefore, Dpp is not a downstream signal of *piwi*. Second, *dpp* overexpression does not rescue *piwi* mutant defects [21]. Therefore, Dpp and niche Piwi are functionally parallel. Third, the *dpp* signaling pathway directly represses *bam* transcription [22, 23]. Likewise, *piwi* niche signaling also downregulates *bam* expression because *bam* is epistatic over *piwi* and because overexpression of Piwi in germarial somatic cells causes overproliferation of GSCs and displaces Bam-C expression beyond region 1 and 2a of the germarium [3]. Taken together, these results indicate that these two signaling pathways must converge at some point to regulate Bam function. The convergence point could be in niche cells,

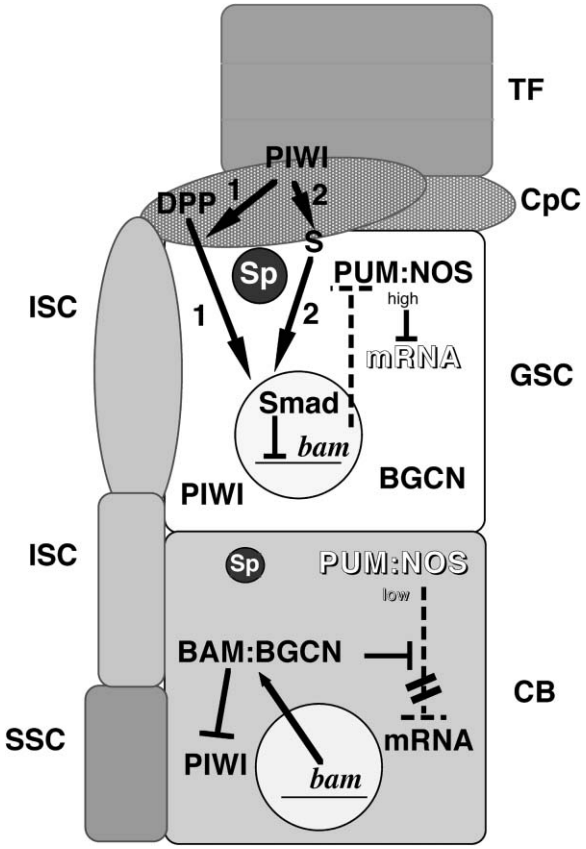

Figure 5. A Model for the Regulation of GSC Self-Renewal versus Differentiation

For simplicity, only the left half of the anterior germarium is depicted. The following abbreviations were used: GSC, germline stem cell; CB, cystoblast; CpC, cap cell-central component of niche cells; TF, terminal filament; ISC, inner sheath cell; SSC, somatic stem cell; Sp, spectrosome; mRNA, unknown differentiation factor suppressed by Pum/Nos; S, unknown signal produced by Piwi; and Smad, dpp transducers. 1 and 2 designate two possibilities for the *dpp* and *piwi* signaling pathways to converge to regulate *bam* function. For a detailed explanation, see text.

where *piwi* directly affects Dpp signal production by aiding in its modifications, stability, and/or secretion (Figure 5, pathway 1). Alternatively, it could be in GSCs, where Piwi suppresses a Dpp agonist(s) or perhaps even the Bam/Bgcn complex (Figure 5, pathway 2). This scenario would require that Piwi produce an intercellular signal independent of Dpp. At present, we cannot differentiate between these two possibilities.

How does Bam function as a converging target in promoting GSC differentiation? It has been suggested that *benign gonial cell neoplasm* (Bgcn) is an obligatory partner for Bam-C as a differentiation factor [24]. Bgcn is expressed in GSCs, but not in somatic cells. This may explain why ectopic *bam* expression only downregulates Piwi in GSCs, but not in somatic cells.

How is Pum involved in the Piwi-Bam pathway? Piwi and Pum do not affect each other's expression, yet *pum* is clearly epistatic to *bam*. This precludes the possibility that Piwi exerts its effect on Bam-C via Pum. The epistasis of *pum* to *bam* is best explained by ascribing a translational repressing function of Pum/Nos in the germline toward mRNAs that promote differentiation. This repression is released by Bam/Bgcn. In GSCs, Bam-C is itself transcriptionally silenced; therefore, Pum and Nos are active in suppressing differentiation. In cystoblasts, Bam/Bgcn are expressed, thereby antagonizing Pum/Nos function. This allows differentiation-promoting mRNAs to be translated. Bgcn is related to the DexH-box family of RNA-dependent helicases [24]. Recently, it has been suggested that the majority of RNA helicases function by displacing proteins from RNA strands rather than by unwinding RNA [25]. It is therefore conceivable that the Bam/Bgcn complex displaces Pum/Nos from their target RNAs.

We propose a model for switching between self-renewal and differentiation of GSCs in the *Drosophila* germarium (Figure 5). The niche cells signal to GSCs by secreting Dpp/Bmp and possibly other proteins. The Dpp signal is received by GSCs through its receptors Punt and Thick Veins (TKV), and it is transduced by pMad to silence *bam* transcription in these cells. This is achieved via the direct binding of Smads to a discrete silencing element in the *bam* gene [22, 23]. Piwi in niche cells has an essential and cooperative function to this signal. Piwi and Dpp signaling pathways converge at some point upstream of *bam*, in either niche cells or GSCs. The absence of Bam allows Pum and Nos to be active, which suppresses the translation of differentiation genes, thus maintaining the stem cell fate. In the cystoblast and differentiating germline cysts, the Dpp signal is no longer effective, thereby relieving the transcriptional repression of *bam*. The Bam/Bgcn complexes then repress Pum/Nos function, allowing these cells to differentiate. Therefore, Pum/Nos can be considered a switch between self-renewal and differentiation, whereas niche signaling through Bam/Bgcn regulates this switch at a single cell level.

Experimental Procedures

Drosophila Strains and Culture

All strains were grown at 25°C on yeast-containing cornmeal molasses/agar medium. The following fly strains were used in this study: The *piwi*^{1} mutant chromosome [1] was dominantly marked with *Irregular facets* (*If*) [26]. The *w;piwi*^{1} *FRT*^{40A} [2] and *y w P[hsFLP]*^{12}*; P[ovo*^{D1}*]*^{2L} *FRT*^{40A} [16] were used for clonal analysis. The *P[myc-piwi]*$^{G38\text{-}2B}$ insertion on the second chromosome was used to visualize Piwi protein expression [3], and is hereafter referred to as *P[myc-piwi]*. *bam*$^{\Delta 86}$ *ry e* is a null allele of the *bam* gene [6]; *P[w*$^{+}$*;hsp70-bam*$^{+}$*]*^{11d} and *P[w*$^{+}$*;hsp70-bam*$^{+}$*]*^{18d} are heat shock inducible transgenes inserted on the wild-type chromosome 3 and X, respectively [7]; the *P[w*$^{+}$ *Bam:GFP]*^{28mc} chromosome bears a full length *bam* gene fused to GFP [22, 27]; *pum*^{ET1} and *pum*^{ET9} are maternal effect null alleles [28] obtained from the Bloomington Stock center; *pum*^{1688} and *pum*^{2003} are hypomorphic alleles with defects in germline stem cell maintenance [4]; *Yb*^{72} is a truncation mutant considered to be a null *Yb* allele [17]. The *en-gal4* and *hs-gal4* transgenes were used to drive niche cell and global overexpression of *EP(2)1024*, respectively. *EP(2)1024* is an EP element insertion, from the original Rorth collection, inserted in the *piwi* gene [3], which hereafter is referred to as *piwi*^{EP}. *Oregon R* served as the wild-type strain in all experiments.

Immunohistochemistry

Ovaries and testes were dissected, fixed, and stained as described in Lin et al. [29]. For immunofluorescence staining, the following antisera were used: rabbit polyclonal anti-VASA antibody (1:2000; [30]); mouse monoclonal anti-1B1 antibody recognizing spectro-

somes and fusomes (1:1; [31]); rabbit polyclonal anti-α-spectrin antibody (1:200; [32]); mouse monoclonal anti-Myc epitope antibody 1-9E10.2 (1:50; [33]); rat polyclonal anti-Bam-C antibody (1:50; [6]); rat polyclonal anti-Pum antibody (1:200; [34]); and rabbit polyclonal anti-GFP antibody (1:200; Molecular Probes). All the fluorescence-conjugated secondary antibodies were from Jackson Immunoresearch Laboratory and were used at 1:200 dilution. Immunofluorescently labeled samples were also counterstained with DAPI, as described previously [29]. Micrographs were taken with either a Zeiss Axioplan microscope or a Zeiss LSM410 confocal microscope, as described in Cox et al. [2].

Bam and Piwi Overexpression Analysis

w^{1118};+/+;P[hsp70-bam$^+$;w$^+$]^{11d}/P[hsp70-bam$^+$;w$^+$]^{11d} males were mated to *w;P[myc-piwi]/P[myc-piwi];+/+* virgin females to analyze the long-term effect of *bam* overexpression on Piwi protein expression. Newly eclosed *w;P[myc-piwi]/+;P[hsp70-bam$^+$;w$^+$]^{11d}/+* females were heat shocked twice daily for 1 hr in a 37°C water bath with a 2 hr recovery period at 25°C between the first and second heat shock treatments. Flies were heat shocked according to this regime for 3 days, and 3 hr after the final heat shock ovaries were dissected and processed for antibody staining.

w^{1118} P[w$^+$;hsp70-bam$^+$]^{18d} virgin females were crossed to *w/Y; P[myc-piwi]/P[myc-piwi]* males to generate *w/w P[w$^+$;hsp70-bam$^+$]^{18d};P[myc-piwi]/+* females to analyze the short-term effects of *bam* overexpression on Piwi protein expression. These females were heat shocked once for 2 hr upon eclosion in a 37°C water bath and then given a 6 hr recovery period at 25°C, at which point ovaries were dissected and processed for antibody staining.

Virgin females expressing a transgene carrying a wild-type *bam* gene fused with GFP, *w^{1118};P[w$^+$;bam:GFP]^{28mc}/P[w$^+$;bam:GFP]^{28mc}; bam$^{\Delta 86}$/bam$^{\Delta 86}$* were crossed to either *w/Y;P[w$^+$;hsp70-myc-piwi]/ P[w$^+$;hsp70-myc-piwi]* males or *wY;piwi^{EP}/CyO;hs-gal4/hs-gal4* males to generate *w^{1118};P[w$^+$;bam:GFP]^{28mc}/+;bam$^{\Delta 86}$/P[w$^+$;hsp70-myc-piwi]* or *w^{1118};P[w$^+$;bam:GFP]^{28mc};piwi^{EP};bam$^{\Delta 86}$/hs-gal4* progeny to analyze the short-term effects of *piwi* overexpression on Bam-C protein expression. Newly eclosed females were heat shocked and processed as described above in this section.

Construction of *bam* Null or *Yb* Null Flies Containing a Transgenic *myc-piwi* Gene

The *w;P[myc-piwi]/CyO;+/+* males were mated to *w;+/+;bam$^{\Delta 86}$ ry e/TM3 Sb ry e* virgin females. Newly eclosed *w;P[myc-piwi]/+; bam$^{\Delta 86}$ ry e/+* males were mated to *w;+/CyO;+/TM3 Sb ry e* virgin females to generate *w;P[myc-piwi]/CyO;bam$^{\Delta 86}$ ry e/TM3 Sb ry e* flies. Sib-matings between males and virgin females established a stock from which *P[myc-piwi]/CyO;bam$^{\Delta 86}$ ry e/bam$^{\Delta 86}$ ry e* females were isolated for fertility tests and for ovary analysis via whole-mount immunofluorescence. The same stock was used to isolate males of the same genotype for fertility and spermatogenesis analysis. Southern analysis of genomic DNA from these flies was performed to verify the presence of the *P[myc-piwi]* transgene and the homozygous *bam* mutant genotype. Similar crosses with *P[myc-piwi]* insertions on the X chromosome and at other sites on second chromosomes were used to verify the results obtained from the transgenic line G38-2B (data not shown).

Yb;P[myc-piwi] flies were constructed by crossing *y Yb^{72} w/FM7 P[act-GFP]* virgins to *w;P[myc-piwi]* males to generate *y Yb^{72} w/Y;P[myc-piwi]/+* males that were crossed to *y Yb^{72} w/FM7 P[act-GFP];Sco/CyO* virgins to generate *y Yb^{72} w//FM7 P[act-GFP];P[myc-piwi]/CyO* flies, which were selected to establish a stock. The *FM7 P[act-GFP]* balancer allowed the discrimination between *Yb* heterozygote versus homozygote larvae.

Double Mutant Analysis

piwi;bam mutant flies were constructed by crossing *piwi^1-If/ CyO;+/+* males to *+/+;bam$^{\Delta 86}$/TM3 Sb* virgin females. *piwi^1-If/+; bam$^{\Delta 86}$/+* males were recovered among the F_1 progeny and mated to *+/CyO;+/TM3 Sb* virgin females. Sib-mating the resulting *piwi^1-If/CyO;bam$^{\Delta 86}$/TM3 Sb* males and virgin females established a stock from which homozygous *piwi^1-If;bam$^{\Delta 86}$* females could be isolated for ovarian analysis by whole-mount immunofluorescence.

Yb;bam double mutant flies were constructed by crossing *y Yb^{72} w/FM6;TM3 Sb e/TM6 Hu Tb e* virgins to *w;bam$^{\Delta 86}$ e/TM3 Sb e* males. The resulting *y Yb^{72} w/Y;bam$^{\Delta 86}$ e/TM3 Sb e or TM6 Hu Tb e* males were recrossed to *y Yb^{72} w/FM6;TM3 Sb e/TM6 Hu Tb e* virgins to generate *y Yb^{72} w/FM6;bam$^{\Delta 86}$ e/TM3 Sb e* flies to establish a stock.

Each *pum* allele was crossed to the *bam*-containing chromosome to generate *pum,bam$^{\Delta 86}$* double mutants. *bam* was selected by its homozygous sterility and verified by PCR. *pum* was selected by its homozygous lethality and verified by the sterility in the *pum^{ET1}/ pum^{ET9}* transheterozygous mutants.

Germline Clonal Analysis of the *piwi^1* Mutant

piwi mutant germline stem cell clones were generated with the FLP-DFS technique [16], as previously described [2]. Because the transgenic *ovo^D* gene on the second chromosome produces highly atrophic germaria ([16]; data not shown), morphologically normal germaria associated with a normal complement of developing egg chambers are derived from homozygous *piwi^1* mutant GSC clones.

Construction of *bam* Mutant Flies Expressing *piwi* in Niche Cells but Not in the Germline

The *w;piwi^{EP}/CyO; bam$^{\Delta 86}$ e/TM3 Sb e* stock was constructed as follows: *w;piwi^{EP}/CyO;TM3 Sb e/TM6 Hu Tb e* were crossed to *w;+/+;bam$^{\Delta 86}$ e/TM3 Sb e* males to generate F_1 *w;+/CyO;bam$^{\Delta 86}$/ TM3 Sb e* virgins, which were mated to their *w/Y;piwi^{EP}/+;bam$^{\Delta 86}$/ TM3 Sb e* sibling males to generate *w;piwi^{EP}/CyO;bam$^{\Delta 86}$ e/TM3 Sb e*, which was recovered by selecting for yellow eyes (*EP*), *Cy e*, and *Sb*. Similarly, *piwi^1* and *bam$^{\Delta 86}$* were combined by crossing the previously recovered F_1 *w;+/CyO; bam$^{\Delta 86}$ e/TM3 Sb e* virgins to *w/Y; piwi^1/CyO;MKRS Sb/TM6 Hu Tb e* males. Single lines were established from the F_2 progeny, and lines that contained sterile non-*Cy* flies (*piwi^1/piwi^1*) were selected because they contained *piwi^1/CyO* instead of *+/CyO*. From these lines, a *w;piwi^1/CyO;bam$^{\Delta 86}$ e/TM6 Hu Tb e* stock was established. In parallel to the above crosses, flies carrying *en-gal4* and *piwi^1* chromosomes were mated to yield *w;en-gal4/piwi^1* females. These virgins were mated to *w/Y;wg^{Sp}/ CyO;MKRS Sb/TM2 Ubx* males. The progeny with pale yellow eyes (*en-gal4*) and *CyO* phenotype were selected (*w,en-gal4,piwi^2/ CyO;+/TM2 Ubx*), and single sib matings were tested for fertility. Sterility of non-*CyO* individuals indicated the presence of an *en-gal4,piwi^1* recombinant chromosome because of the presence of *piwi^1* in both parents. Lines that also contained third chromosome balancers *w,en-gal4,piwi^1/CyO;+/TM2 Ubx* or *MKRS Sb* were used to introduce *bam$^{\Delta 86}$*. *w;piwi^1/CyO; bam$^{\Delta 86}$ e/TM6 Hu Tb e* virgins were mated to *w,en-gal4,piwi^1/CyO;+/TM2 Ubx* or *MKRS Sb* males, and flies with pale yellow eyes (*en-gal4*) and not *TM6 Hu Tb* balancer (*bam$^{\Delta 86}$*) were selected to establish a stock. The experimental crosses were *w;piwi^{EP}/CyO;bam$^{\Delta 86}$ e/TM3 Sb e* virgins mated to either *w/Y;en-gal4,piwi^1/CyO;bam$^{\Delta 86}$ e/MKRS Sb* or *w/Y;piwi^1/CyO; bam$^{\Delta 86}$ e/MKRS Sb*.

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